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Remarks:

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) **A novel signal transduction factor and a gene encoding the same**

(57) A novel signal transduction factor and a gene encoding the same are provided. The present novel signal transduction factor has, for example, the amino acid sequence set forth in SEQ ID NO: 4, and the DNA sequence encoding the factor has, for example, the sequence set forth in SEQ ID NO: 1.

**EP 0 874 046 A1**

## Description

The present invention relates to a novel gene encoding one of proteins, called Smad, which intracellularly transduce stimuli elicited by a physiologically active substance belonging to the transforming growth factor beta (hereinafter referred to as TGF $\beta$ ) family.

The TGF $\beta$  family is a group of peptidic physiologically active substances widely distributed in the animal kingdom. This family include very important physiologically active peptides such as TGF $\beta$  found as a substance involved in proliferation of tumor cells, bone morphogenetic protein (BMP) that has a significant function for bone formation in vertebrates including human, inhibin that regulates the secretion of follicle-stimulating hormone from pituitary gland, activin that possesses activity as an erythroide differentiation factor, and neurotrophic factor derived from glial cells (Bock, G. R. and Marsh, J. eds., 1991, Clinical Application TGF $\beta$ , Ciba Foundation Symposium, Johns Wiler & Sons). A clue to solution to the question how the TGF $\beta$  family peptides act on cells was initially obtained by cDNA cloning of receptors for these peptides and by determining their nucleotide sequences together with the amino acid sequences deduced therefrom. Receptors for this family have a transmembrane-type protein-phosphotransferase activity (protein kinase activity) specific to serine (and threonine) residues. This fact demonstrates that phosphorylation of intracellular protein is involved in transduction of stimuli from the TGF $\beta$  family (Sporn, M. B. and Roberts, A. B. eds., 1990, "Peptide Growth Factors and Their Receptors", part I and II, Springer-Verlag, Berlin).

As factors mediating stimuli from the TGF $\beta$  family, genes designated as Mad and Sma are hitherto known in *Drosophila* and *Nematoda*, respectively. In recent years, several genes showing homology to Mad and Sma have been found in several vertebrates including human. Their cDNAs have been cloned and their nucleotide sequences were determined. Those genes and the proteins encoded by the same were named Smad, and to date, Smad1, Smad2, Smad3, Smad4, Smad5, and Smad6 have been reported (Derynck R. *et al.*, 1996, "Nomenclature: Vertebrate mediators of TGF $\beta$  family signals", *Cell*, **18**, 173). Furthermore, during early embryogenesis, Smad1 is known to be essential for a basic and significant determination as for which side of the embryo becomes dorsal and the other becomes ventral (Graff, J. M. *et al.*, 1996, *Cell*, **85**, 479-487). It has been shown that inactivation of Smad2 gene is one of the causes for colorectal cancer in human, while Smad4 gene is shown to be identical to a tumor suppressor gene DPC4 that is known to be strongly associated with repression of pancreatic cancer (Eppert, K., *et al.*, 1996, *Cell*, **86**, 543-552; Hahn, S. A. *et al.*, 1996, *Science*, **271**, 350-353). Thus, the Smad protein family may be signal transduction factors which transduce stimuli from physiologically active peptides of the TGF $\beta$  family, while they may also be factors profoundly involved in generation of cancer.

The known Smad family proteins are intracellular proteins consisting of about 400-550 amino acid residues which have an amino- and a carboxy-terminal regions relatively well conserved among the family. As a consequence of increased kinase activity of the specific receptors induced by the TGF  $\beta$  family stimulus, Smad proteins are rapidly phosphorylated and concentrated into nucleus. In the nucleus, the field of gene transcription, Smad proteins uniquely regulate gene expression through unknown mechanism mediated by oligomer formation among the same or different kinds of molecules (Massagu J., 1996, *Cell*, **85**, 947-950).

In recent years, it has been turned out that a variety of physiologically active substances like TGF $\beta$ , including hormones and cytokines, function eventually through regulation of gene expression in the target cells. Specificity of activity of each physiologically active substance is determined by the nature of receptor and subsequent signal transduction factor for the particular substance. In addition, a signal caused by a single physiologically active substance often activate several kinds of signal transduction factors, which results in branching of the transduction pathway. Isolation of signal transduction factors and elucidation of their properties are therefore helpful to understand mechanisms through which various physiologically active substances function, and to employ the factors as targets for pharmaceuticals.

As described above, the TGF $\beta$  family members play very important roles in various physiological events including growth control, immune response, cell differentiation, morphogenesis during embryo and the like. More than 50 physiologically active substances belonging to the TGF $\beta$  family are hitherto known, and they include substances of which deficiency or excess in quantity, or abnormality in quality is known to be associated with pathologies related to the above physiological events, such as cancer, autoimmune disease, osteoporosis, anemia, congenital deformity and the like. Similarly, genetic analyses have also shown that defects in the Smad family, which transduces stimuli (signals) from the TGF $\beta$  family, is involved in various abnormalities or pathologies, for example, in cancer which is the highest cause of death in advanced countries including many of Europe and North America. For prevention or treatment of cancer, it is desirable to elucidate all of the genes associated with cancer. However, relatively small number of Smads have been hitherto identified when compared with the already known physiologically active substances belonging to the TGF $\beta$  family. This fact suggests that there remain unidentified Smad family members. Therefore, isolation of a novel Smad gene will enable us to find a further pathway involving the TGF $\beta$  family, and such a gene is expected to be useful as a diagnostic agent for detecting abnormalities, such as tumor, at gene level.

The present invention aims to provide a novel factor belonging to the Smad family which transduces a signal of a physiologically active substance of the TGF $\beta$  family, and to provide a gene encoding said factor.

In view of the important role, in biological responses, of TGF $\beta$  peptides and their signal transducer Smad family proteins, the present inventors screened cDNAs derived from *Mus musculus* in order to clone a novel Smad gene. As a result, cDNA clones which correspond to mRNA encoding a novel Smad family protein were identified in a cDNA mixture derived from the whole tissue of the 17-day embryos. The present invention has been completed on the basis of this finding.

Specifically, the first object of the present invention is to provide a gene encoding a novel signal transduction factor which belongs to the Smad family.

The second object of the present invention is to provide a protein encoded by the above gene, that is, a signal transduction factor.

#### BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 is a gene map of an expression vector pactEF-Smad7 for expression of Smad7 in animal cells.

Fig. 2 shows the result of denaturing polyacrylamide gel electrophoresis of a fused protein between Smad7 and Myc-tag peptide.

Fig. 3 is a gene map of a plasmid vector pIBL-mSmad7 in which Smad7 has been cloned.

Fig. 4 shows the result of agarose gel electrophoresis of mRNA (sense-strand RNA) and antisense RNA for Smad7.

Fig. 5 shows the result of denaturing polyacrylamide gel electrophoresis of Smad7 protein synthesized *in vitro*.

Fig. 6 shows a sequence comparison between Smad7 and Smad1.

#### (1) A gene encoding a signal transduction factor Smad7

The novel Smad in the present invention (Smad7) has the following characteristics.

##### 1) coding region

As shown in SEQ ID NO: 1, it consists of 1281 nucleotide pairs, and encodes a sequence consisting of 426 amino acid residues shown in SEQ ID NO: 4.

##### 2) 5' terminal non-coding region

It comprises 209 nucleotide pairs shown in SEQ ID NO: 2, and the coding region described in 1) is contiguously linked to its 3' end.

##### 3) 3' terminal non-coding region

It comprises 207 nucleotide pairs and is linked immediately to the coding region described in 1).

The cDNA for the novel Smad of the present invention (Smad7) was obtained by the procedures as described below. Firstly, a highly homologous region was determined among the amino acid sequences of vertebrate Smad family members already reported. The amino acid sequence of such highly homologous region is expected to be essential for important function of the Smad family, and therefore, it must be conserved also in unknown Smads. Accordingly, oligonucleotide primers for DNA amplification by PCR (polymerase chain reaction) method were designed and synthesized (Saiki, R. *et al.*, 1985, *Science*, **230**, 1350-1354) on the basis of the information about the highly homologous region. A pool of cDNA mixture which was prepared using a publicly known method (Kenji Okazaki, 1995, "mRNA-No-Chousei-Hou", Shunsuke Uda *et al.* eds., *In* "Meneki-Jikken-Sousa-Hou", vol I, pp. 349-352, Nankodou) from polyadenylated RNAs derived from *Mus musculus* embryos and which was ligated to an adapter DNA was used as a template source in the PCR. The PCR was performed using the primer oligonucleotide described above in combination with an oligomer specific to the adapter DNA. The partial amino acid sequences deduced from the nucleotide sequence of a PCR product was recognized to have a homology with the amino acid sequence of the Smad family proteins. Based on the nucleotide sequence thus obtained, oligomers corresponding to the 5' and 3' termini were synthesized, and used in the PCR in which the above cDNA mixture was used as a template source to obtain a cDNA containing the entire coding region. After cloning of this cDNA in a general-purpose plasmid vector, the nucleotide sequence was determined. Since the nucleotide sequence of this cDNA is now shown in SEQ ID NOs: 1, 2 and 3, one can also obtain this cDNA by synthesizing sense and antisense oligomers respectively corresponding to the 5'- and 3'-termini of the DNA, and then performing a PCR in which a *Mus musculus* embryo cDNA mixture is used as a template source.

When the amino acid sequence deduced from the cDNA sequence so obtained was compared with the known amino acid sequence of Smad1 (NCBI (U.S. National Center for Biotechnology Information) Identification numbers:

1332714, 1333647, 1381671, 1518645, and 1654323), it was revealed that the sequences exhibit relatively high homology of 65% in the C-terminal region, demonstrating that the obtained cDNA encodes a Smad family protein. In addition, the amino acid sequence is apparently different from the sequence of any one of the previously disclosed vertebrate-derived Smad family members, Smad1 - Smad6 (NCBI Identification: Smad1; 1518645, 1658159, 1333647, 1654323, 1469308, 1438077, 1332714: Smad2; 1407782, 1575530: Smad3; 1673577, 1552532: Smad4; 1724091, 1163234: Smad5; 1518647, 1654325: Smad6; 1654327) and from the invertebrate-derived *Drosophila* Mad (NCBI Identification: 1085150, 551489) and Nematoda Sma (NCBI Identification: 1173452, 1173453, 1173454). Thus, the present cDNA was identified as cDNA for a novel Smad family protein and designated as Smad7. The nucleotide sequence of the present cDNA is also distinctly different from any one of previously disclosed Smad family cDNAs (GenBank accession numbers: Smad1; U54826, U57456, U58992, U59912, U59423, U58834, L77888: Smad2; U59911, U60530, U65019, U68018, L77885: Smad3; U68019, U76622: Smad4; U79748, U44378: Smad5; U58993: Smad6; U59914).

## (2) Smad7 protein

The Smad7 protein of the present invention has the following characteristics.

### 1) Amino acid sequence

The amino acid sequence deduced from the above Smad7 cDNA nucleotide sequence is shown in SEQ ID NO: 4.

### 2) Molecular weight

The molecular weight of Smad7 protein calculated from the amino acid sequence shown in SEQ ID NO: 4 is 46516.

### 3) Isoelectric point

The isoelectric point of Smad7 protein calculated from the amino acid sequence shown in SEQ ID NO: 4 is 8.3.

Smad7 protein was obtained by the procedures as described below.

Smad7 cDNA shown in SEQ ID NO: 1 was ligated downstream to a promoter region in a known expression plasmid vector, and the vector was transformed into *E. coli* cells, from which the plasmid DNA was then purified. The plasmid DNA was then introduced into cultured cells to produce Smad7 protein. As a vector for the expression of Smad7, pactEF-mSmad7 (Fig. 1) constructed by the present inventors was used. *E. coli* strain transformed with this vector, *Escherichia coli* (pactEF-mSmad7), has been deposited with National Institute of Bioscience and Human Technology (deposition date: April 8, 1997; accession number: FERM P-16187). Smad7 protein can be synthesized in *E. coli* cells transformed with Smad7 cDNA. There are many known vectors for expression in *E. coli* cells, and one can construct a desirable expression vector by inserting Smad7 cDNA into one of those known vectors. Such known vectors are, for example, pET series vectors, pKC30, and the like (Sambrook, J. *et al.*, 1989, "Molecular Cloning", Cold Spring Harbor Laboratory Press, USA).

In addition, a fusion protein in which the entire or a part of Smad7 protein is fused to another amino acid sequence can also be expressed. To this end, a gene or oligonucleotide encoding an amino acid sequence to be added is ligated to the entire or a part of Smad7 cDNA so that the codon frames for both sequences agree with each other.

Furthermore, the Smad7 cDNA shown in SEQ ID NO: 1 may be transcribed into RNA, and the RNA so obtained may be added to an intracellular translation system or cell-free translation system to synthesize Smad7 protein. There are many known vectors for transcription of cloned DNA into RNA. For example, SP64, pIBI31, pGEM3 and the like may be used for this purpose (Sambrook, J. *et al.*, 1989, "Molecular Cloning", Cold Spring Harbor Laboratory Press, USA). As RNA polymerases, those derived from bacteriophages SP6, T3 and T7 may be used. As a system for synthesizing protein from synthesized sense-strand RNA, a system using oocytes of *Xenopus laevis* is known (Mayumi Nishizawa, Noriyuki Sakata, 1992, "in vitro-No-Tanpakushitu-No-Seigousei", In "Shin-Seikagaku-Jikken-Kouza, 1, Tanpakushitu VI", edited by The Japanese Biochemical Society, Tokyo Kagaku Dojin). Similarly, a method using rabbit reticulocyte lysate may also be used as a cell-free translation system (Kozak, M., 1990, *Nuc. Acids Res.*, **18**, 2828). In another embodiment, the Smad7 cDNA shown in SEQ ID NOs: 1, 2 and 3 or a part thereof may be used as a template to synthesize an antisense RNA for Smad7. Such antisense RNA may be used for diagnosis of Smad7-related pathologies. Furthermore, by ligating DNA having an appropriate sequence to a transcription vector containing a sequence for Smad7, antisense RNA may be synthesized as RNA molecule having a ribozyme activity.

According to known techniques, one skilled in the art can obtain mutant proteins in which deletion, substitution or insertion of one or more amino acid residues are introduced into the amino acid sequence shown in SEQ ID NO: 4, by introducing mutation(s) into the DNA shown in SEQ ID NO: 1 in the Sequence Listing, for example, by a site-directed mutagenesis. Among such mutant proteins, those retaining a signal transduction activity are included within the scope

of the present invention.

## EXAMPLES

The present invention is further illustrated by the following Examples.

### Example 1: Cloning of *Mus musculus* Smad7 cDNA

#### 1) Design of primer oligonucleotide and PCR

A gene-specific antisense oligomer GSP1 (Gene Specific Primer 1) having a sequence:

5'-GTT(A/C/G/T)A(A/G)GTG(A/C/G/T)AC(C/T)TC(A/C/G/T)A(G/T)CCAGCA(A/C/G/T)GG-3'

was synthesized on the basis of the following amino acid sequence:

Pro Cys Trp Leu/Ile Glu Val/Ile His Leu Asn which is well conserved among Smad proteins. Similarly, another gene-

specific antisense oligomer GSP2 having a sequence:

5'-GTA(A/C/G/T)(C/T)(A/C)(A/C/G/T)G(C/G)(A/C/G/T)CCCCA(A/C/G/T)CC(C/T)TT(A/C/G/T)AC(A/G)AA-3'

was synthesized on the basis of the following amino acid sequence:

Phe Val Lys Gly Trp Gly Ala/Pro/Cys/Glu Thr.

The first PCR was performed using adapter oligonucleotide-attached cDNA mixture derived from *Mus musculus*

17-day embryo (manufactured by CLONTECH, USA) as a template pool, with the following adapter-specific oligomer 1 :

(AP1): 5'-CCATCCTAATACGACTCACTATAGGGC-3'

as a sense oligomer and the above GSP1 as an antisense oligomer. The reaction conditions were as follows: after 180 seconds at 96°C, 5 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 72°C for 240 seconds, 5 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 70°C for 240 seconds, and then 25 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 68°C for 120 seconds.

Then, DNA molecules contained in 1/500 volume of the reaction mixture so obtained was used as templates in the second PCR in which the following adapter-specific oligomer 2:

(AP2): 5'-ACTCACTATAGGGCTCGAGCGGC-3'

and the above GSP2 were used as sense and antisense oligomers, respectively. The reaction conditions were as follows: after 2 minutes at 96°C, 20 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 68°C for 120 seconds. In these PCR, Expand High Fidelity PCR System (BOEHRINGER MANNHEIM, Germany) (Barnes, W. M., 1994, *Proc. Natl. Acad. Sci. USA*, **91**, 2216-2220) was used as thermostable DNA polymerase, and PCR Thermal Cycler MP (TAKARA SHUZO) was used as a heat cycle equipment.

Analysis of the nucleotide sequence of about 1,200 bp DNA fragment obtained in the above PCR revealed that it contained both sequences encoding the following two amino acid sequences conserved among the Smad family,

Lys Lys Leu Lys Glu, and

Arg Trp Pro Asp Leu.

The DNA fragment was, therefore, considered to be derived from cDNA encoding a Smad family protein.

#### 2) Analysis of the 5' and 3' terminal regions

On the basis of the nucleotide sequence obtained in the above item 1), the following Smad-specific antisense oligomer:

5'-CCGGACGAGCGCAGATCGTTTGGTCC-3'

was synthesized in order to perform a 5'-RACE (rapid amplification of 5'-cDNA ends) (Frohman, M.A., 1993, *Methods. Enzymol.*, **218**, 340-358). Similarly, the following Smad-specific sense oligomer:

5'-TTCATGCAGCAACCATGGACGGGTTTC-3'

was also synthesized in order to perform a 3'-RACE (rapid amplification of 3'-cDNA ends) (Frohman, M. A., 1993, *Methods. Enzymol.*, **218**, 340-358). In these reactions, the same cDNA mixture derived from *Mus musculus* 17-day embryos as that used in the above item 1) was used as template source, together with AP2 and the Smad-specific antisense oligomer for 5'-RACE, or together with the Smad-specific sense oligomer and AP2 for 3'-RACE. The reaction conditions were as follows: after 180 seconds at 96°C, 5 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 72°C for 240 seconds, 5 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 70°C for 240 seconds, and then 25 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 68°C for 240 seconds.

Nucleotide sequencing of about 250 bp DNA fragment obtained in this 5'-RACE revealed that it contains an initiation codon located at the position conserved among the Smad family and the 5' non-coding region shown in SEQ ID NO: 2. Similarly, nucleotide sequencing of about 400 bp DNA fragment obtained in the above 3'-RACE revealed that it

contains an termination codon and the 3' non-coding region shown in SEQ ID NO: 3.

### 3) Cloning of Smad7 coding region

On the basis of the nucleotide sequence determined in the above item 2), the following specific sense oligomer (to which an *NheI* site for cloning has been added at the 5'-end):

5'-CCGCTAGCACCATGTTTCAGGACCAAACGATCTGCGCTCGTC-3'

and the following antisense oligomer (to which a *Bam*HI site for cloning has been added at the 5'-end):

5'-CCGGATCCTATCGCGAGTTGAAGATGACCTCCAGCCAGCAGC-3'

were prepared. With these two oligomers, PCR was performed using the same cDNA mixture as that described in the above item 1) as template source. The reaction conditions were as follows: after 180 seconds at 96°C, 5 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 72°C for 60 seconds, 5 cycles of denaturation at 96°C for 30 seconds and annealing/elongation at 70°C for 60 seconds, and then 25 cycles of denaturation at 96°C for 30 seconds, annealing/elongation at 68°C for 60 seconds.

About 0.5 µg of about 1,300 bp DNA fragment obtained in the above reaction was treated with restriction enzymes *NheI* (New England Biolabs, USA) and *Bam*HI (New England Biolabs, USA) at 37°C for one hour, and then purified by gel electrophoresis in 0.7% low-melting point agarose. The DNA fragment was then cloned using a known plasmid vector pIBIΔ (International Biotechnologies, Inc., USA; it may be prepared from pIBI31 according to the method of Furuno, N. *et al.*, 1994, *EMBO J.*, **13**, 2399-2410) and host cells, JM109, derived from *E. coli* K-12 strain (Sambrook, J. *et al.*, 1989, "Molecular Cloning", Cold Spring Harbor Laboratory Press, USA) (TOYOBO, Japan) to obtain a plasmid pIBIΔ-mSmad7 (Fig. 3). *E. coli* strain transformed with this plasmid, *Escherichia coli* (pIBIΔ-mSmad7) has been deposited at National Institute of Bioscience and Human Technology (deposition date: April 8, 1997; accession number: FERM P-16188). The deposition was converted to the international deposition under Budapest Treaty on March 30, 1998, and assigned new accession number FERM BP-6317. This plasmid DNA was then isolated, and the nucleotide sequence of the cDNA was determined. The nucleotide sequence is shown in SEQ ID NO: 1. Furthermore, Smad7 cDNA having the sequence shown in SEQ ID NO: 1 was also obtained from *Mus musculus* 11-day embryos using the identical procedures.

#### Example 2: Construction of Smad7 expression vector

The Smad7 cDNA obtained above, which contains the entire coding region, was inserted into an expression vector for cultured animal cells, pactEF (BOEHRINGER MANNHEIM, Germany; it may be prepared from pEMBL9 (+) according to the method of Okazaki K. and Sagata, N., 1995, *EMBO J.*, **14**, 5048-5059), to construct pactEF-mSmad7 (Fig. 1). As above, *E. coli* strain transformed with this vector, *Escherichia coli* (pactEF-mSmad7), has been deposited with National Institute of Bioscience and Human Technology (deposition date: April 8, 1997; accession number: FERM P-16187). The deposition was converted to the international deposition under Budapest Treaty on March 30, 1998, and assigned new accession number FERM BP-6316. In Fig. 1, β-Actin promoter/EF-1α enhancer means a transcription promoter of beta-actin derived from chicken genome and the elongation factor 1 alpha derived from human genome; f1 ori means the DNA replication initiating region of f1 phage; bla means a beta-lactamase gene (conferring sulbenicillin resistance and ampicillin resistance); ori means the DNA replication initiating region derived from pUC plasmid; poly A means transcription termination and polyadenylation signal derived from SV40; Smad7 means Smad7 cDNA; *Hind*III, *Ban*III, *Scal*, and *Bam*HI are the sites at which the DNA is cleaved by respective restriction enzymes.

In addition, a vector pactEF-Myc-mSmad7 was also prepared for expression of the fusion protein in which a fragment containing the following sequence of a known epitope peptide, Myc-tag (Evan, G. I. *et al.*, 1985, *Mol. Cell. Biol.* **5**, 3610-3616):

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu

has been added to the N-terminus of Smad7. *E. coli* strain transformed with this vector, *Escherichia coli* (pactEF-Myc-mSmad7), has been deposited with National Institute of Bioscience and Human Technology (deposition date: April 8, 1997; accession number: FERM P-16186). The deposition was converted to the international deposition under Budapest Treaty on March 30, 1998, and assigned new accession number FERM BP-6315. To prepare this vector, the following DNA oligomer:

5'-ATGTCTGAGCAGAAGCTGATCTCTGAGGAAGACCTTGGAGCTAGCACC-3'

was inserted just before the translation initiation codon of Smad7. This vector DNA was then introduced into mouse NIH3T3 cells by the calcium phosphate method (Graham, F. L. and van der Eb, A. J., 1973, *Virology*, **52**, 456-457). After 48 hours, the whole cell extract was separated by denaturing polyacrylamide gel electrophoresis (Laemmli, U. K. *et al.*, 1970, *J. Mol. Biol.*, **49**, 99-113), and analyzed by immunoblotting (Harlow, E. and Lane D., 1988, "Antibodies", Cold Spring Harbor Laboratory Press, USA) using anti-Myc-tag monoclonal antibody (SANTA CRUZ, USA). The electrophoretic analysis revealed expression of the fused protein between Smad7 and Myc-tag as a band at the position cor-

responding to a molecular weight of about 48,000 (Fig. 2). In Fig. 2, Lane M shows molecular weight markers; Lane 1 shows the fused protein between Smad7 and Myc-tag peptide synthesized in animal cells.

### Example 3: Synthesis of Smad7 RNA

The vector pBlA into which the Smad cDNA was cloned in Example 1 contains a promoter sequence for phage T7 RNA polymerase upstream to the cDNA, and also a promoter sequence for phage T3 RNA polymerase downstream to the cDNA (Furuno, N. *et al.*, 1994, *EMBO J.*, **13**, 2399-2410). Therefore, in order to obtain a sense-strand RNA of Smad7, 2 µg of the plasmid pBlA-mSmad7 (Fig. 3) described in Example 1, which was obtained by cloning Smad7 cDNA into pBlA, was treated with restriction enzyme *Bam*HI at 37°C for one hour, and the linearized plasmid so obtained was then subjected to a transcription step using T7 RNA polymerase (Ambion, USA). The synthesized RNA was gel-electrophoretically homogeneous (Fig. 4). Similarly, 2 µg of the above cDNA was treated with restriction enzyme *Hind*III at 37°C for one hour, and the linearized plasmid so obtained was then subjected to a transcription step using T3 RNA polymerase (Ambion, USA). The synthesized antisense RNA was gel-electrophoretically homogeneous (Fig. 4). In Fig. 3, f1 ori means the DNA replication initiating region of f1 phage; bla means a beta-lactamase gene (conferring sulbenicillin resistance and ampicillin resistance); ori means the DNA replication origin region derived from a pUC plasmid; pT7 means a promoter sequence for phage T7 RNA polymerase; Smad7 means Smad7 cDNA; pT3 means a promoter sequence for phage T3 RNA polymerase; *Fsp*I, *Ssp*I, *Scal*, *Hind*III, *Bam*HI, *Eco*RI and *Aat*I are the sites at which the DNA is cleaved by respective restriction enzymes. In Fig. 4, Lane 1 shows Smad7 mRNA (sense-strand RNA), and Lane 2 shows Smad7 antisense RNA.

### Example 4: Synthesis of Smad7 protein

About 1 µg of the Smad7 sense-strand RNA obtained in Example 3 was added to a cell-free lysate derived from rabbit reticulocytes (Promega, USA) together with <sup>35</sup>S-labeled amino acids (Amersham, UK), and the mixture was subjected to translation at 30°C for one hour to obtain Smad7 protein. Denaturing polyacrylamide gel electrophoresis of the product along with molecular weight markers revealed that the synthesized Smad7 was a homogeneous protein having molecular weight of about 47,000 (Fig. 5). In Fig. 5, Lane M shows molecular weight markers; Lane 1 shows the Smad7 protein; Lane 2 shows 2-fold amounts of the Smad7 protein as compared with Lane 1; Lane 3 shows 4-fold amounts of the Smad7 protein as compared with Lane 1; and the arrowhead indicates the position of the Smad7 protein.

As shown in Fig. 6, the amino acid sequence of the novel factor of the present invention, Smad7, contains a region highly homologous to the previously known signal transduction factor Smad1. The highly homologous region is also well conserved in other Smad family proteins, suggesting that it is a region essential for the Smad activity. It is, therefore, believed that Smad7 having this region retains the function as a signal transduction factor.

### Example 5

Using the Smad7 protein as antigen, which was prepared from the expression vector obtained by the procedures described above, antisera specific to Smad7 protein may be obtained by immunizing rabbits according to a known method (Harlow, E. and Lane D., 1988, "Antibodies", Cold Spring Harbor Laboratory Press, USA). The antisera so obtained may be further affinity-purified by using Smad7 protein as affinity ligand according to a known procedure (Harlow, E. and Lane D., 1988, "Antibodies", Cold Spring Harbor Laboratory Press, USA) to obtain inhibitory antibody highly specific to Smad7 protein. This inhibitory antibody may be added to a reaction mixture in order to assay the activity of Smad7. Furthermore, this inhibitory antibody may be micro-injected into living cells by a known method (Capecchi, M., 1980, *Cell*, **22**, 479-488) in order to confirm the signal transduction activity in the cells. Similarly, the vector pactEF-mSmad7 DNA described above (Fig. 1) may also be directly injected into cells in order to assay the activity of the expressed product, *i.e.* Smad7 protein.

Thus, the novel signal transduction factor of the present invention and the gene encoding the same are useful as a pharmaceutical or diagnostic agent.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Biomolecular Engineering Research Institute
- (B) STREET: 6-2-3, Furuedai, Suita-shi
- (C) CITY: Osaka
- (E) COUNTRY: JP
- (F) POSTAL CODE (ZIP): none

(ii) TITLE OF INVENTION: A novel signal transduction factor and a gene encoding the same

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 98 10 7432.1

## (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: JP 105892/1997
- (B) FILING DATE: 23-APR-1997

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1281 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mus musculus
- (B) STRAIN: Swiss-Webster/NIH

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGTTTCAGGA CCAAACGATC TGCGCTCGTC CGGCGTCTCT GGAGGAGCCG TGCGCCCGGC	60
GGCGAGGACG AGGAGGAGGG CGTGGGGGGT GGC GCGGAG GAGGCGAGCT GCGGGGAGAA	120
GGGGCGACGG ACGGCCGGGC TTATGGGGCT GGTGGCGGCG GTGCGGGCAG GGCTGGCTGC	180
TGCCTGGGCA AGGCAGTCCG AGGTGCCAAA GGTCAACCACC ATCCCCATCC CCCAACCTCG	240
GGTGCCGGGG CGGCCGGGG CGCCGAGGCG GATCTGAAGG CGCTCAGCA CTCGGTGCTC	300
AAGAAACTCA AGGAGCGGCA GCTGGAGCTG CTGCTTCAGG CCGTGGAGTC CCGCGGCGGT	360



# EP 0 874 046 A1

ACGCGCACCG CGTGCCTCCT GCTGCCCCGC CGCCTGGACT GCAGGCTGGG CCCGGGGGCG 420

CCCGCCAGCG CGCAGCCCGC GCAGCCGCCC TCGTCTACT CGTCCCCCT CCTGCTGTGC 480

5 AAAGTGTCA GGTGGCCGGA TCTCAGGCAT TCCTCGGAAG TCAAGAGGCT GTGTTGCTGT 540

GAATCTTACG GGAAGATCAA CCCCAGAGCTG GTGTGCTGCA ACCCCCATCA CCTTAGTCGA 600

CTCTGTGAAC TAGAGTCTCC CCCTCCTCCT TACTCCAGAT ACCCAATGGA TTTTCTCAAA 660

10 CCAACTGCAG GCTGTCCAGA TGCTGTACCT TCCTCCGCGG AAACCGGGGG AACGAATTAT 720

CTGGCCCCTG GGGGGCTTTC AGATTCCCAA CTCTTCTGG AGCCTGGGGA TCGGTCACAC 780

TGGTGCGTGG TGGCATACTG GGAGGAGAAG ACTCGCGTGG GGAGGCTCTA CTGTGTCCAA 840

GAGCCCTCCC TGGATATCTT CTATGATCTA CCTCAGGGGA ATGGCTTTTG CCTCGGACAG 900

CTCAATTCCG ACAACAAGAG TCAGCTGGTA CAGAAAGTGC GGAGCAAGAT CGGCTGTGGC 960

20 ATCCAGCTGA CGCGGAAGT GGATGGCGTG TGGGTTTACA ACCGCAGCAG TTACCCCATC 1020

TTCATCAAGT CCGCCACACT GGACAACCCG GACTCCAGGA CGCTGTTGGT GCACAAAGTG 1080

TTCCCTGGTT TCTCCATCAA GGCTTTTGAC TATGAGAAAG CCTACAGCCT GCAGCGGCCC 1140

25 AATGACCACG AGTTCATGCA GCAACCATGG ACGGGTTTCA CCGTGCAGAT CAGCTTTGTG 1200

AAGGGCTGGG GCCAGTGCTA CACCCGCCAG TTCATCAGCA GCTGCCCGTG CTGGCTGGAG 1260

GTCATCTTCA ACAGCCGGTA G 1281

30 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 209 base pairs

(B) TYPE: nucleic acid

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mus musculus

(B) STRAIN: Swiss-Webster/NIH

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

45 CGGCGCCCGC GCGCGCCCCG GCCTCTGGGA GACTGGCGCA TGCCACGGAG CGCCCCCTCGG 60

GCCGCCGCCG CTTCTGCCCC GGCCCCTGCT GTTGCTGCTG TCGCCTGCGC CTGCTGCCCC 120

50 AACTCGGCGC CCGACTTCTT CATGGTGTGC GGAGGTCATG TTCGCTCCTT AGCCGGCAAA 180

CGACTTTTCT CCTCGCCTCC TCGCCCCGC 209

55

## (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 207 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Mus musculus  
 (B) STRAIN: Swiss-Webster/NIH

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
TCGGTCGTGT GGTGGGGAGA AGAGGACAGG GCGGATCGTG AGCCGAGCAG GCCCCCGTTC      60
AAACTACTTG CTGCTAACCT TTCCCGAGTG ATTGCTTTTC ATGCAAACTC TTTGGTTGGT      120
GTTGTTATTG CCATTCATTG TTGGTTTTGT TTTGTTCTGT TCTGGTTTGT TTTTTTTTTT      180
TTTTTCCTCC TCCTTTCTCG TTAAAAA                                           207
```

## (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 426 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Mus musculus  
 (B) STRAIN: Swiss-Webster/NIH

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Phe Arg Thr Lys Arg Ser Ala Leu Val Arg Arg Leu Trp Arg Ser      15
 1           5           10           15
Arg Ala Pro Gly Gly Glu Asp Glu Glu Gly Val Gly Gly Gly Gly      30
 20           25           30
Gly Gly Gly Glu Leu Arg Gly Glu Gly Ala Thr Asp Gly Arg Ala Tyr      45
 35           40           45
Gly Ala Gly Gly Gly Gly Ala Gly Arg Ala Gly Cys Cys Leu Gly Lys      60
 50           55           60
Ala Val Arg Gly Ala Lys Gly His His His Pro His Pro Pro Thr Ser      80
65           70           75           80
```

EP 0 874 046 A1

	Gly	Ala	Gly	Ala	Ala	Gly	Gly	Ala	Glu	Ala	Asp	Leu	Lys	Ala	Leu	Thr	
					85					90					95		
5	His	Ser	Val	Leu	Lys	Lys	Leu	Lys	Glu	Arg	Gln	Leu	Glu	Leu	Leu	Leu	
				100					105					110			
	Gln	Ala	Val	Glu	Ser	Arg	Gly	Gly	Thr	Arg	Thr	Ala	Cys	Leu	Leu	Leu	
			115					120					125				
10	Pro	Gly	Arg	Leu	Asp	Cys	Arg	Leu	Gly	Pro	Gly	Ala	Pro	Ala	Ser	Ala	
		130					135					140					
	Gln	Pro	Ala	Gln	Pro	Pro	Ser	Ser	Tyr	Ser	Leu	Pro	Leu	Leu	Leu	Cys	
	145					150					155					160	
15	Lys	Val	Phe	Arg	Trp	Pro	Asp	Leu	Arg	His	Ser	Ser	Glu	Val	Lys	Arg	
				165						170					175		
	Leu	Cys	Cys	Cys	Glu	Ser	Tyr	Gly	Lys	Ile	Asn	Pro	Glu	Leu	Val	Cys	
				180					185					190			
20	Cys	Asn	Pro	His	His	Leu	Ser	Arg	Leu	Cys	Glu	Leu	Glu	Ser	Pro	Pro	
			195					200					205				
	Pro	Pro	Tyr	Ser	Arg	Tyr	Pro	Met	Asp	Phe	Leu	Lys	Pro	Thr	Ala	Gly	
		210					215					220					
25	Cys	Pro	Asp	Ala	Val	Pro	Ser	Ser	Ala	Glu	Thr	Gly	Gly	Thr	Asn	Tyr	
	225					230					235					240	
	Leu	Ala	Pro	Gly	Gly	Leu	Ser	Asp	Ser	Gln	Leu	Leu	Leu	Glu	Pro	Gly	
				245					250						255		
30	Asp	Arg	Ser	His	Trp	Cys	Val	Val	Ala	Tyr	Trp	Glu	Glu	Lys	Thr	Arg	
			260						265					270			
	Val	Gly	Arg	Leu	Tyr	Cys	Val	Gln	Glu	Pro	Ser	Leu	Asp	Ile	Phe	Tyr	
		275						280					285				
35	Asp	Leu	Pro	Gln	Gly	Asn	Gly	Phe	Cys	Leu	Gly	Gln	Leu	Asn	Ser	Asp	
		290				295						300					
	Asn	Lys	Ser	Gln	Leu	Val	Gln	Lys	Val	Arg	Ser	Lys	Ile	Gly	Cys	Gly	
	305				310						315					320	
40	Ile	Gln	Leu	Thr	Arg	Glu	Val	Asp	Gly	Val	Trp	Val	Tyr	Asn	Arg	Ser	
				325					330					335			
	Ser	Tyr	Pro	Ile	Phe	Ile	Lys	Ser	Ala	Thr	Leu	Asp	Asn	Pro	Asp	Ser	
			340					345						350			
45	Arg	Thr	Leu	Leu	Val	His	Lys	Val	Phe	Pro	Gly	Phe	Ser	Ile	Lys	Ala	
		355					360					365					
	Phe	Asp	Tyr	Glu	Lys	Ala	Tyr	Ser	Leu	Gln	Arg	Pro	Asn	Asp	His	Glu	
	370				375							380					
50	Phe	Met	Gln	Gln	Pro	Trp	Thr	Gly	Phe	Thr	Val	Gln	Ile	Ser	Phe	Val	
	385				390						395					400	
55																	

Lys Gly Trp Gly Gln Cys Tyr Thr Arg Gln Phe Ile Ser Ser Cys Pro  
405 410 415

Cys Trp Leu Glu Val Ile Phe Asn Ser Arg  
420 425

## Claims

1. A protein having a signal transduction activity which comprises the amino acid sequence shown in SEQ ID NO: 4, or a mutant of said protein retaining the signal transduction activity, which is obtained by introducing into said amino acid sequence a deletion, substitution or insertion of one or more amino acid residues.
2. A nucleic acid encoding the amino acid sequence shown in SEQ ID NO: 4.
3. A nucleic acid of claim 2 which comprises the DNA sequence shown in SEQ ID NO: 1.
4. An expression vector comprising the nucleic acid of claim 2 or 3.
5. A transformant containing the expression vector of claim 4.
6. A DNA comprising the DNA sequence shown in SEQ ID NO: 2.
7. A DNA comprising the DNA sequence shown in SEQ ID NO: 3.
8. Pharmaceutical composition comprising the protein of claim 1 or the nucleic acid of claim 2 or 3 and optionally a pharmaceutically acceptable carrier.
9. Diagnostic composition comprising the protein of claim 1 or the nucleic acid of claim 2 or 3.

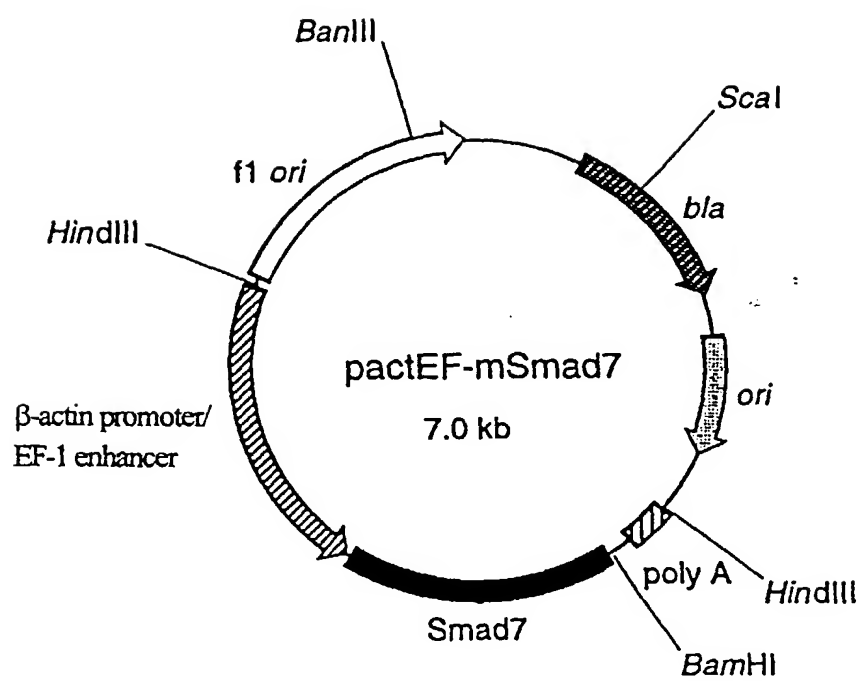


Fig. 1

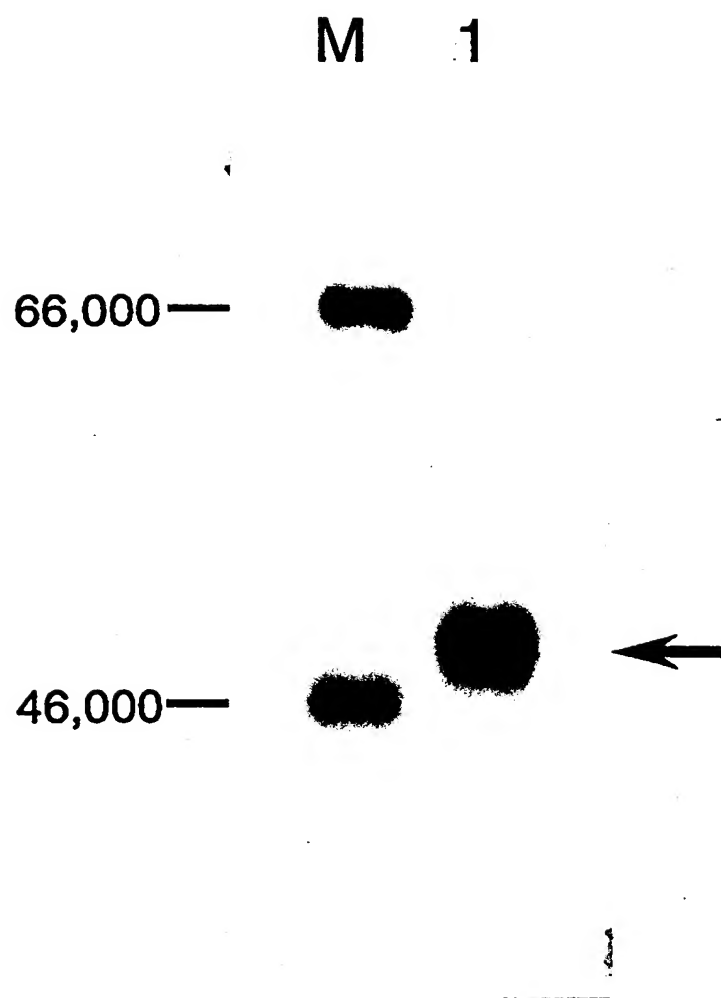


Fig. 2

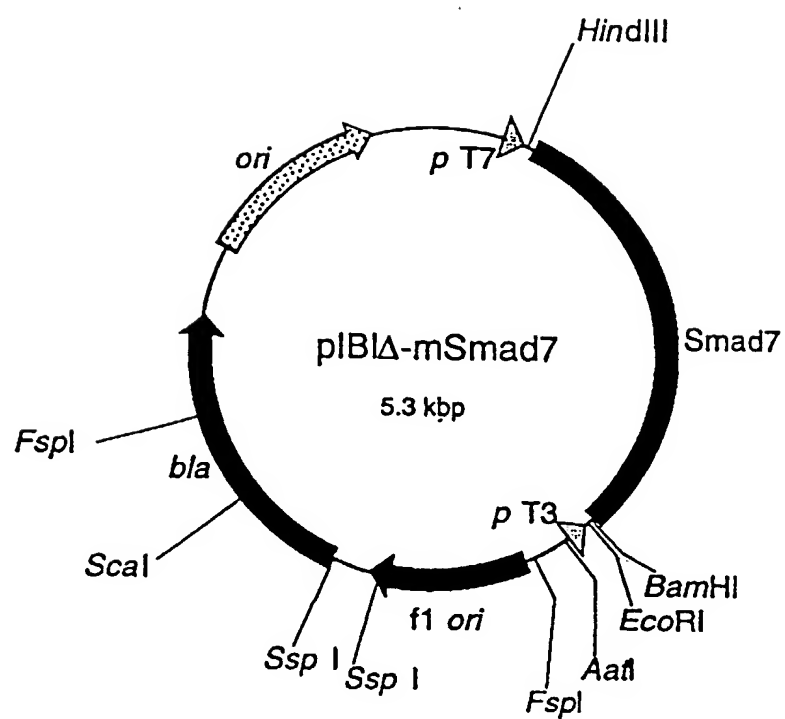


Fig. 3



Fig. 4



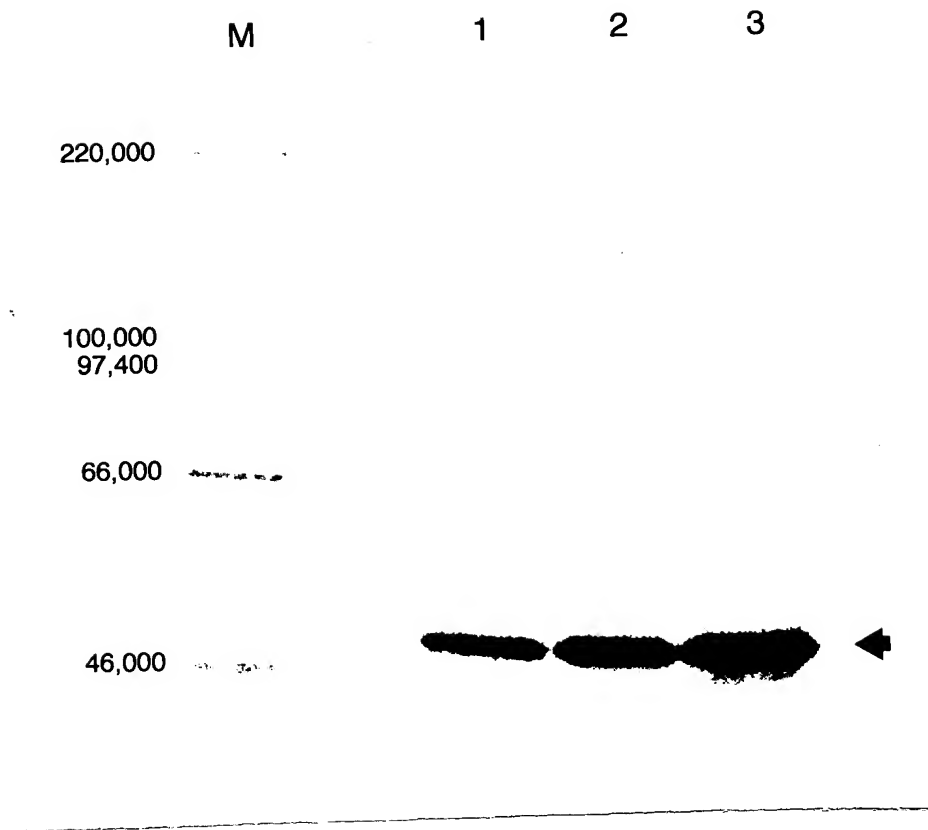


Fig. 5

Smad7	1: MFR TK-----RSALVRR LRWSRAPGGDEEEGVGGGGGGLRGEGVTDGRAYGAGGGGAGRAGCCLGKAVRGAKGHRHPHPPTSGAGAAG	86
Smad1	1: MNV TSLF SFTSPAVKRLL-----GWKQGDEEEKW-----*	29
Smad7	87: GAEADLKAL THSVLKKLKERQ-LELLQAVESRGGTRTACLLLPGR LDCRLPGPAPASAQPAQPPSSYSLP LLLCKVFRWPDLRHSSEVK	175
Smad1	31: -AE---KAV-DALVKKLKKKGAMEELEKALSCPGQPSNCVTIPRSLDGR L-----QVSHRKGLPHV---IYCRVNRWPD LQSHHELK	104
Smad7	176: RLCCCESYGIKINPELVCCNP HLSRLCE-----	203
Smsd1	105: PLECC EFPFGSKQKEVCTNPYHYKRVESPLPV LVPRHSEYNPQHSLLAQFRNLGQNEPHMPLNATFPD SFQQPN SHPFP HSPNSSYPN	194
Smad7	204: -----LESPPPPYSRYPMDFLKPTAGCDPAVSSSVETGGTNYLAPGGLSDSQ LLLLEPGDRSHWCYVAYWE	268
Smad1	195: SPGSSSSTYPHSPTSSDPGSPFQMPADTPPPAYL-PPEDPMT-QDGSQPMDTNMA PPLPSEINRG----DVQAVA-YEEP KHWCSIVVYE	278
Smad7	269: EKTRVGRLYCVQEPS-L-DIFYDL PQG-NGFCLGQLNSDNKSQ LVQKVR SKIGCGTQLTREVDGWMVYNRSSYP IFIKSATLD-NPDSRT	354
Smad1	279: LNNRVGEAFHASSTSVLDGFTDPSNNKNRFLGLLSNVNRNSTIENTRRHIGKG VHLYYVGGEWYAECLSDSSIFVQSRNCNYHHGFHP	368
Smad7	355: LLVHKVFPGF SIKAF-DYEKAYS LQRPNDHEFMQ-PWTFG-FTVQISFVKGWGQC YTRQFISSCPWLEVIFNSR-----	426
Smad1	369: TTVCKIPSGCSLKIFNNQEF AQLLAQSVNHGFETVYELTKMCTIRMSFVKGWGAETHRQDVTSTPCWIEHLHGPLQWLDKVL TQMGSPH	458
Smad7	:-----	
Smad1	459: NPISSVS	465



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 98 10 7432

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
P,X	WO 97 30065 A (MILLENNIUM PHARMACEUTICALS INC) 21 August 1997 * the whole document * ---	1,8,9	C12N15/12 C12N1/21 C07K14/435 A61K38/17 C12Q1/68
P,X	WO 97 39105 A (UNIV JOHNS HOPKINS) 23 October 1997 * the whole document * ---	1,8,9	
A	NAKAO,A. ET AL.: "Identification of Smad2, a human Mad-related protein in the transforming growth factor beta signaling pathway" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 5, January 1997, pages 2896-2900, XP002070780 * abstract * ---	1	
A	ZHANG,Y. ET AL.: "The tumor suppressor Smad4/DPC4 as a central mediator of Smad function" CURRENT BIOLOGY, vol. 7, no. 4, March 1997, pages 270-276, XP002070781 * abstract * -----	1	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C12N C07K A61K C12Q
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 8 July 1998	Examiner Hillenbrand, G
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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